A Novel TCEP Removal Method for the Synthesis of Site-specific Antibody Drug Conjugates

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Abstract

Antibody drug conjugates (ADCs) are an emerging class of biopharmaceutical drug designed to treat cancer. They are comprised of monoclonal antibodies (mAbs) which are conjugated (linked) to potent drugs. The monoclonal antibodies provide high levels of specificity and are responsible for identifying and binding to antigens present on cancerous cells. After binding, antibody drug conjugate complexes are internalized by the cell. Once inside, the drug is released and the cell is killed. Thus, antibody drug conjugates are designed to kill only cancerous cells, leaving healthy tissue largely unaffected. This is very different from traditional chemotherapy methods that non discriminately kill both rapidly dividing cancerous and healthy cells causing many side effects.

Currently, the synthesis of antibody drug conjugates requires many steps and is time consuming. For the purposes of this project, we focused on one specific synthesis step in the process called dialysis. During the synthesis process, the mAb must be reduced using a small molecule called Tris(2carboxyethyl)phosphine hydrochloride (TCEP). After reduction, TCEP is no longer necessary, but still in solution with the mAb. In order to successfully make antibody drug conjugates, this TCEP must be separated from the mAb. The current process to do this on a laboratory scale setting is called dialysis. Dialysis exploits the size difference between TCEP and mAb to achieve separation. We have identified this as one of the longest steps in the synthesis process.

We hypothesized that we can use column chromatography in flow through mode as an alternative method to separate TCEP from mAb – TCEP mixtures. This method also exploits the size difference between TCEP and mAb to achieve separation. We tested microporous resins that allow small molecules like TCEP, but not large molecules like mAb to enter the pores. After identifying top performing resins, we went through the ADC synthesis process but implemented chromatography instead of dialysis. We concluded that this new method does remove TCEP from solution and is a promising alternative to dialysis.

Table of Contents

Introduction

Antibody drug conjugates (ADCs) are an important class of cancer therapeutic. These molecules are made up of three components, a monoclonal antibody (mAb), a drug and a linker.[1] They have a highly specific mechanism of action, and are designed so that treatment is localized at the site of the tumor, while healthy tissue is largely unaffected. This is very different from the current standard of non selective chemotherapy, which kills rapidly dividing cells, regardless of whether they are cancerous or not.

Currently, the synthesis of antibody drug conjugates is a lengthy multi – step process. In this project, we focused on one step of the process called dialysis. This step is used to purify and separate the mAb from a small molecule called Tris(2carboxyethyl)phosphine hydrochloride (TCEP) and is necessary for optimal ADC synthesis. Our project investigates whether an alternative, more efficient method called column chromatography in flow through mode can achieve comparable results.

Antibody Drug Conjugates

Antibody drug conjugates are comprised of three components, a monoclonal antibody (mAb), a drug and a linker.[1] (Figure 1) The mAb is responsible for finding and binding to specific antigens expressed on cancer cells. The drug is a cytotoxic agent designed to kill cancer cells. Finally, the linker is responsible for attaching the antibody to the drug.

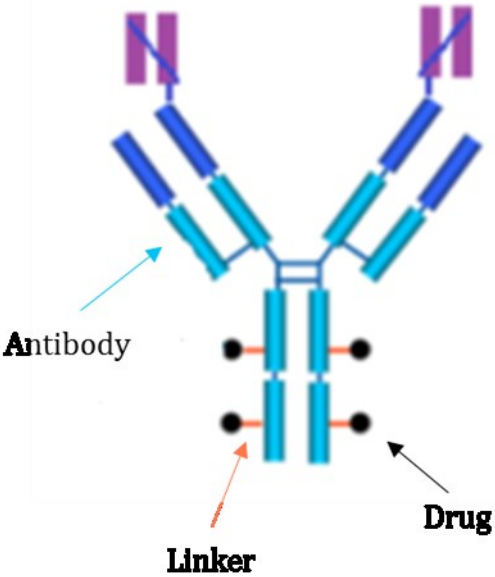

Figure 1: Antibody drug conjugate structure: antibody, linker and drug [2]

ADC Mechanism of Action

Antibody drug conjugates are designed to kill cancer cells specifically at tumor sites. Initially, ADC's are released into the body and circulate until mAbs identify and bind to an antigen receptor expressed on a cancer cell.[3] (Figure 2) After binding, the ADC complex undergoes receptor – mediated endocytosis and enters the cell. Once inside the cell, the antibody and linker are degraded by the lysosome. Concurrently, the drug is released into the cell and causes cell death. Other cancer cells in the vicinity may also be killed through a process called the bystander effect.[4] The mechanism of cancer cell death is dependent on drug type. There are two very prevalent types of drug.[5] The first blocks tubulin assembly, which in turn prevents cell replication. The second binds to and damages DNA.

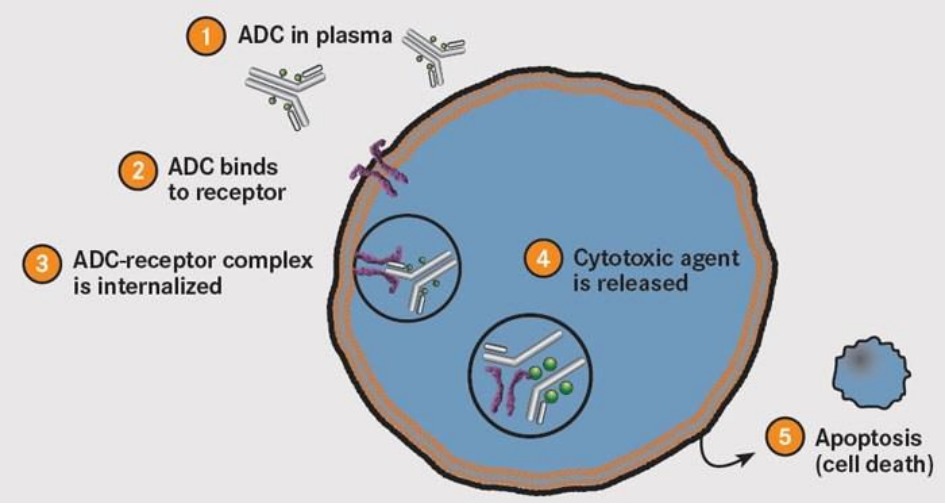

Figure 2: Antibody drug conjugate mechanism of action [6]

ADC Synthesis

This project involves a class of ADCs known as a site – specific antibody drug conjugates. With this type of ADC, drug load and conjugation site are specified. During the synthesis process, a cysteine is engineered into the mAb. [7] This cysteine is capped with

glutathione via a disulfide bond during expression. However, we need to attach the mAb to the linker and drug at this engineered cysteine site. Thus, the glutathione cap must be removed. The process to do this is called reduction. (Figure 3)

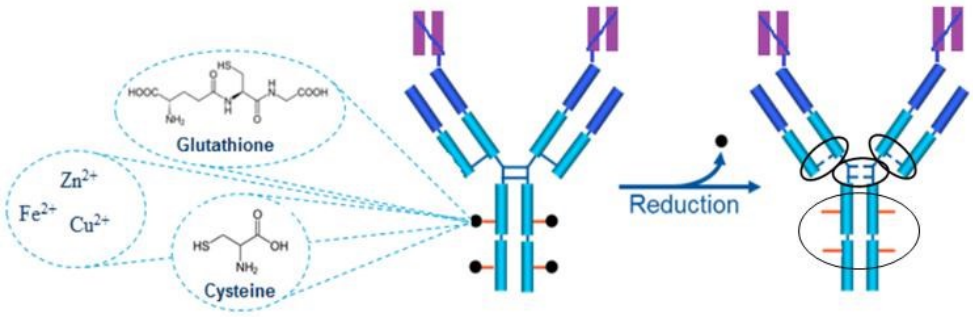

Figure 3: Antibody drug conjugate synthesis, reduction of disulfide bonds[2]

Currently, reduction is performed using a small molecule called Tris(2carboxyethyl)phosphine hydrochloride (TCEP). (Figure 4) The purpose of reduction is to remove the glutathione cap from cysteine. This is done by reducing disulfide bonds and introducing free thiol groups.[7] (Figure 5) The antibody is then attached to the linker and drug at these free thiol groups.

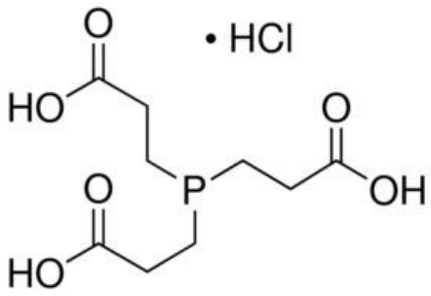

Figure 4: TCEP structure [8]

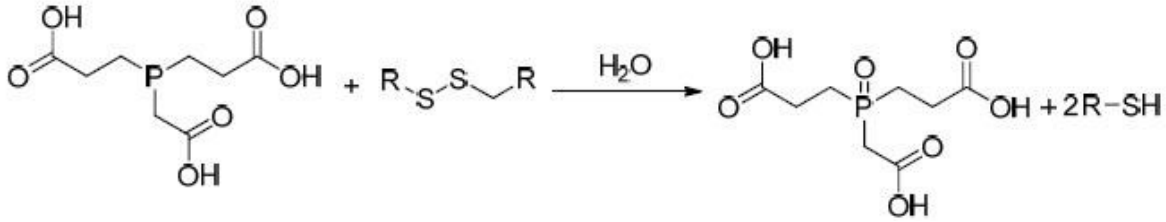

Figure 5: Reduction of disulfide bridges with TCEP [9]

However, after reduction, TCEP is no longer necessary, but still in solution with the mAb. In the subsequent synthesis steps, the presence of TCEP causes unwanted side

reactions and results in lower conjugation efficiency. Thus, it is important to remove TCEP from the mAb-TCEP solution. The current process to do this and focus of our project is called dialysis.

Dialysis removes TCEP from the sample and also dilutes it with buffer. For example, it is common for mAb concentration to go from 10 mg/ml to 5 mg/ml during this process. To get back to the initial concentration we have to concentrate the sample. After this, the mAb undergoes reoxidation of disulfide bridges. In the previous reduction step, TCEP is used to reduce the disulfide bonds in the mAb to introduce free thiols. This is necessary at the engineered cysteine site where we want to connect the linker and drug to the mAb. However, in the process, other disulfide bonds in the mAb are also reduced (Figure 6). To reform these bonds, the mAb must undergo oxidation. After oxidation, the final step is conjugation, which attaches the linker and drug to the mAb.

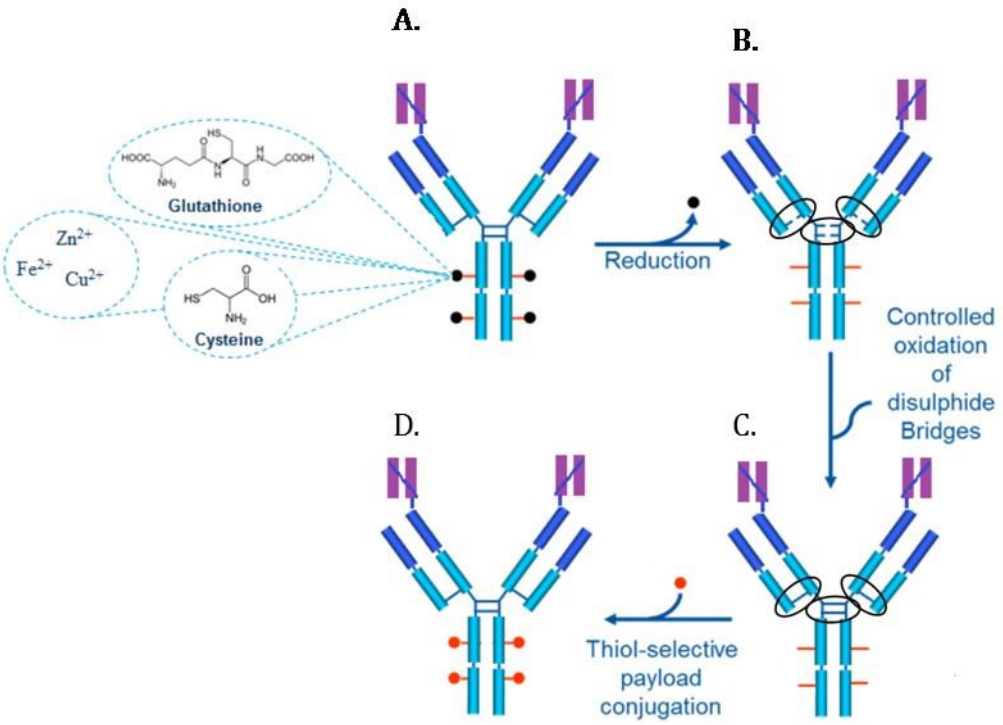

Figure 6: ADC Synthesis: (A) antibody intermediate (B) antibody intermediate after reduction (C) antibody intermediate after sample concentration and controlled oxidation (D) antibody intermediate after thiol-selective payload conjugation[2]

Dialysis

For small scale synthesis of antibody drug conjugates, dialysis is used to remove TCEP from mAb-TCEP solutions. This separation method exploits the size difference between TCEP and mAbs. For reference, TCEP is 287 grams/mole while the mAb is 210,000 grams/mole. Dialysis involves placing the mAb-TCEP sample in a semi – permeable dialysis membrane, which is then placed in buffer. The small TCEP molecules diffuse out of the membrane while the mAb remains inside and separation is achieved.[10]

These dialysis membranes are characterized by their molecular-weight cutoff (MWCO). The MWCO is the smallest average molecular mass of a standard molecule that will not effectively diffuse across the membrane. For example, a membrane with a 3K MWCO generally retains >90% of a protein with a molecular mass of at least 3 kDA or 3,000 grams/mol. Thus, we can use the molecular weights of TCEP and mAb to determine a MWCO that will ensure maximum separation. Currently, the dialysis process takes ~6+ hours.

Chromatography

We believe we can use column chromatography in flow through mode as an alternative method to separate TCEP from mAb-TCEP solutions. In this process, a chromatographic column is packed with a resin. This resin contains beads and these beads contain pores. For efficient separation, we require a resin with beads that will retain TCEP. Furthermore, the pores of these beads need to be small enough that the mAb cannot enter, and instead must go around.[11] Thus, separation is achieved. (Figure 7)

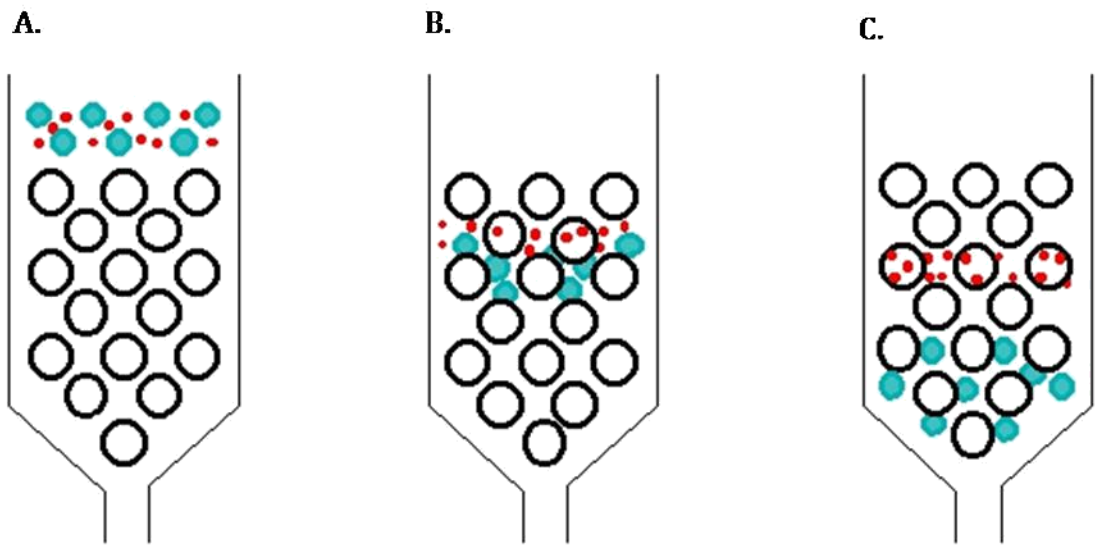

Figure 7: Separation using microporous resins (A) The mAb-TCEP sample is placed into a column filled with microporous resin (B) TCEP molecules become trapped in the beads while the mAb is too large to enter (C) mAb-TCEP separation is achieved[12]

We are primarily interested in microporous anion exchange resins. According to the manufacturer, these resins generally have a microporosity less than 30 Angstroms. Furthermore, these resins carry a positive charge. We believe that negatively charged TCEP molecules will bind to these resins at a high degree, while the mAb will not. Furthermore, we believe this process will take ~1 hour, which is substantially less than dialysis.

Dynamic Binding Capacity (DBC)

In order to achieve efficient separation, we must identify chromatographic resins that bind to TCEP but don't bind to our mAb. We use dynamic binding capacity (DBC) to measure this property. This is the binding capacity under operating conditions (i.e. in column under specified conditions) and is defined as the amount of target molecule that binds to the medium (resin) under given flow conditions before a significant breakthrough of molecule occurs.[13] For our process, we require a resin with a high binding capacity for TCEP but low binding capacity for mAb.

Experimental Methods

Dynamic Binding Capacity of TCEP

Initial experiments were carried out to test the dynamic binding capacity of TCEP on multiple resins. All experiments were carried out using an AKTA Pure, GE Healthcare. Uv-vis measurements on the AKTA Pure were taken at 195 nm. All experiments were performed using Omnifit columns, Diba Industries. All resins were manufactured by Dow Chemical. TCEP was prepared in a 20mM Phosphate Buffer.

Control

For this experiment, the system was on bypass mode and the sample did not pass through a column. The method conditions and buffers are as follows:

	Sample/Buffer	Volume (mL)	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	2
Sample Load	2 mM TCEP	10	2
Equilibration	20 mM Phosphate	8	2

Amberlite FPX 66

A 1.6 ml column was packed with Amberlite FPX 66 resin, a macroporous non-functionalized adsorbent.[14] The method conditions and buffers are as follows.

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	2
Sample Load	2 mM TCEP	30	2
Regeneration	2% Methanol	10	.5
Column wash	Water for injection (WFI)	5	.5

*1 Column volume = 1.6 ml

Similar experiments were performed using Amberlite FPA 98Cl, Amberlite XAD 7HP, and Dowex 1x4.[15, 16, 17] Method conditions and buffers can be found in Appendix A.

Dynamic Binding Capacity of TCEP Using Microporous Resins

Further experiments were carried out to test the dynamic binding capacity of TCEP on microporous resins at multiple pH levels. All experiments were carried out using an AKTA Pure, GE Healthcare. Uv-vis measurements on the AKTA Pure were taken at 195 nm. All experiments were performed using Omnifit columns, Diba Industries.

Dowex 1x4, Dowex 1x8 and Dowex Marathon A2 resin were manufactured by Dow Chemical.[17. 18] Experiments with these resins were performed at pH 6, 6.5 and 7. Diaion SK1B resin was manufactured by Mitsubishi Chemical Corporation.[19] Experiments with this resin were performed at pH 7. pH adjustments were made using 1M Tris and 6N HCl buffer. TCEP was prepared in a 20mM Phosphate Buffer.

Dowex 1x4

A 1.9 ml column was packed with Dowex 1x4 resin. The method conditions and buffers are as follows.

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	2
Sample Load	2 mM TCEP	5.32	2
Equilibration	20 mM Phosphate	10	2
Regeneration	.1 N NaOH	5	1.31
Column wash	20 mM Phosphate	5	.4

*1 Column volume = 1.9 ml

Method conditions and buffers for experiments using Dowex 1x8, Dowex Marathon A2 and Diaion SK1B can be found in Appendix B.

Dynamic Binding Capacity of mAb1

Experiments were carried out to test the dynamic binding capacity of mAb1 on microporous resins at multiple pH levels . All experiments were carried out using an AKTA Pure, GE Healthcare. Uv-vis measurements on the AKTA Pure were taken at 280 nm. All experiments were performed using Omnifit columns, Diba Industries.

Experiments with Dowex 1x4, Dowex 1x8 and Dowex Marathon A2 resin were performed at pH 6, 6.5 and 7. The experiment with Diaion SK1B resin was performed at pH 7. The control experiment was performed at pH 7. pH adjustments were made using 1M Tris and 1N Acetic Acid buffer. TCEP was prepared in a 20mM Phosphate Buffer.

Control

For this experiment, the system was on bypass mode and the sample did not pass through a column. The method conditions and buffers are as follows.

	Sample/Buffer	Volume (mL)	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	5 mg/ml mAb1	2	.3
Equilibration	20 mM Phosphate	10	1.31

Dowex 1x4

A 1.9 ml column was packed with Dowex 1x4. The method conditions and buffers are as follows.

For pH 7 and 6.5

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	5.66 mg/ml mAb1	1	.3
Equilibration	20 mM Phosphate	10	1.31
Regeneration	.1 N NaOH	5	1.31
Column Wash	20 mM Phosphate	5	1.31

*1 Column volume = 1.9 ml

For pH 6

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	4.53 mg/ml mAb1	1.2	.3
Equilibration	20 mM Phosphate	10	1.31
Regeneration	.1 N NaOH	5	1.31
Column Wash	20 mM Phosphate	5	1.31

*1 Column volume = 1.9 ml

Method conditions and buffers for experiments using Dowex 1x8, Dowex Marathon A2 and Diaion SK1B can be found in Appendix C.

Dynamic Binding Capacity of TCEP-mAb1 Solution

An experiment was carried out to test the dynamic binding capacity of a TCEP-mAb1solution on Dowex 1x4 resin. This experiment was carried out using an AKTA Pure, GE Healthcare. Uv-vis measurements on the AKTA Pure were taken at 195 nm and 280 nm. This experiment was performed using an Omnifit column, Diba Industries packed with 1.9 ml of resin. This experiment was run at pH 6.5 and pH adjustments were made with 1M Acetic Acid. TCEP was prepared in a 20mM Phosphate Buffer.

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	4.44 mg/ml mAb1, 2mM TCEP	1.2	.3
Equilibration	20 mM Phosphate	10	1.31
Regeneration	.1 N NaOH	5	1.31
Column Wash	20 mM Phosphate	5	1.31

*1 Column volume = 1.9 ml

ADC Synthesis

A final experiment synthesizing the antibody drug conjugate was performed to test the success of column chromatography as an alternative to dialysis.

Control experiments:

A control experiment was performed using the current ADC synthesis method, as described below. A negative control experiment was also performed following the method described below. However, during the negative control, the sample did not undergo the dialysis and sample concentration steps.

1. Antibody preparation
   mAb1 stock protein at 20 mg/ml was diluted to 10 mg/ml with 1X PBS, pH 7.2. Then the pH was adjusted to 6.8 - 7.2 with 1M Tris buffer.

2. Reduction

   TCEP was added to the solution at 40 mole equivalents of TCEP to 1 mole of antibody. The pH was adjusted to 6.5 - 7 with 1M Tris buffer. After, the solution was stirred at room temperature for two hours.

3. Dialysis

   The sample was filtered using a .2 um PES membrane, ThermoFisher Scientific and transferred to a 10K Slide-A-Lyzer Dialysis Cassette, ThermoFisher Scientific . Then, the membrane was transferred to 1X PBS, pH 7.2 buffer. The solution was stirred at 4 °C for three hours and then the membrane was transferred to new solution of 1X PBS, pH 7.2 buffer. This new solution was stirred at 4 °C overnight.

4. Sample concentration

   The sample was removed from the membrane and filtered using a .2 um PES membrane, ThermoFisher Scientific. It was then concentrated to 10 mg/ml using a Vivaspin 6 MWCO 10000 Sample Concentrator, GE Healthcare.

5. Oxidation

   DHAA was added to the solution at 20 mole equivalents of DHAA to 1 mole of antibody. The solution was stirred for three hours at room temperature.

6. Conjugation

   DMSO was added to the solution at 10% of the conjugation volume. The payload was added to the solution at 7.5 mole equivalents of payload to 1 mole of antibody. The solution was stirred for one hour at room temperature.

7. Quenching of conjugation

   N-acetyl Cysteine (NAc) was added to the solution at 4 mole equivalents of NAc to 1 mole of drug-linker. This solution was stirred for thirty minutes at room temperature. The sample was filtered using a .2 um PES membrane, ThermoFisher Scientific.

8. Analysis

   Analysis of the sample was performed using reverse phase high performance liquid chromatography on an Agilent system. See Appendix D for method specifications.

ADC Synthesis with Column Chromatography

This experiment followed the same procedure as the control, however, the dialysis step was replaced with a column chromatography step. Method conditions and buffers for experiments with Dowex 1x4, Dowex 1x8, Dowex Marathon A2, Diaion SK1B and the control can be found in Appendix E.

ADC Synthesis with Column Chromatography and no Sample Concentration

Negative Control without sample concentration

This experiment generally followed the same procedure as the control described above however there were three changes.

1. In the sample preparation step mAb1 was diluted to 5 mg/ml
2. The dialysis step was removed
3. The sample concentration step was removed.

Column chromatography without sample concentration

This experiment generally followed the same procedure as the control described above however there were three changes.

1. In the sample preparation step mAb1 was diluted to 5 mg/ml
2. The dialysis step was replaced with a column chromatography step
3. The sample concentration step was removed.

Method conditions and buffers for experiments with Dowex 1x8, Dowex Marathon A2, Diaion SK1B and the control can be found in Appendix F.

Results

The results from the AKTA Pure and Agilent system came in the form of chromatograms. Data from the AKTA Pure indicated the dynamic binding capacity of TCEP, mAb1 and mAb1-TCEP mixtures while data from the Agilent showed successful vs failed ADC synthesis.

Dynamic Binding Capacity of TCEP

Generally, a numerical amount of binding is determined by finding sample concentration using the Beer-Lambert Law,

$$A = \varepsilon CL,$$

A = absorbance
ε = molar extinction
coefficient C = concentration
L = path length

However, in this case, the molar extinction coefficient of TCEP was unknown. As an alternative method of identifying whether TCEP was binding to the resins, we used the control experiment as a baseline. The control indicated the maximum absorbance increase of the system (UV detector) if no binding occurred. After adding 10 ml of 2 mM TCEP we found this value to be 500 mAU Thus, if the maximum absorbance increase of a resin is near 500 mAU then we know it is not binding to TCEP.

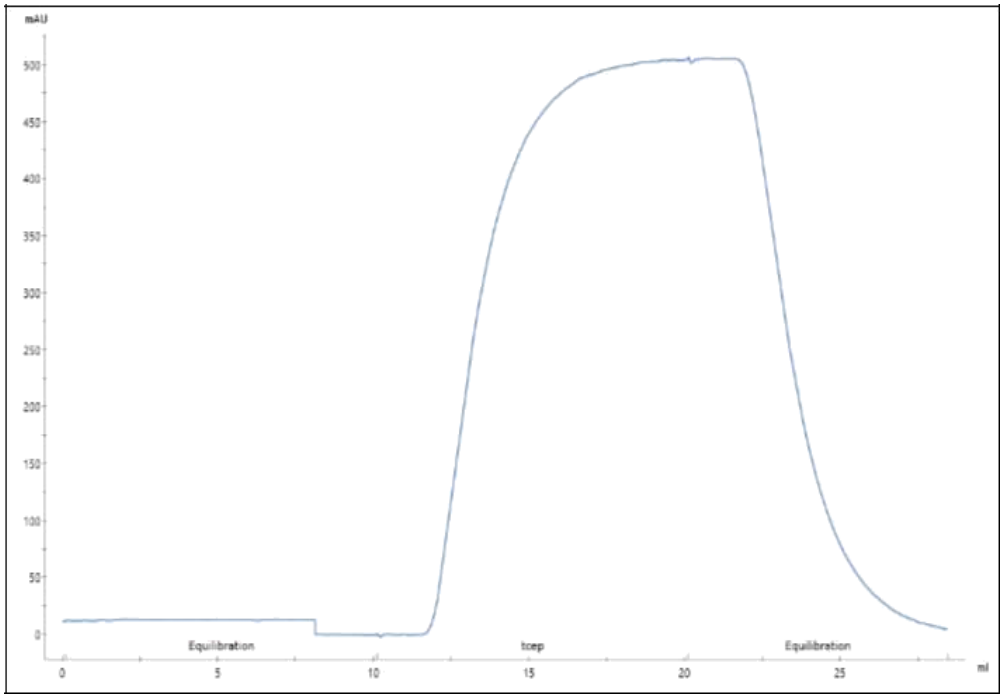

Figure 8: Chromatogram of control, TCEP, maximum absorbance increase of 500 mAU

Using the results from the control experiment, and Table 1, it is clear that Amberlite FPX66 and Amberlite XAD 7HP did not bind to TCEP. While the maximum absorbance increase using these resins was higher than the control, we assumed they were within the margin of error for this experiment. Amberlite FPA 98Cl bound to TCEP more tightly than Amberlite FPX66 and Amberlite FPA 98Cl, however, the binding was still not considered strong. Dowex 1x4 resin outperformed all other resins and bound tightly to TCEP.

Table 1: Maximum Absorbance Increase of Amberlite and Dowex Resins (TCEP)

Resin	Maximum Absorbance Increase (mAU)	Amount of 2 mM TCEP added to reach maximum absorbance increase (ml)
Amberlite FPX 66	575	13
Amberlite FPA 98Cl	275	24
Amberlite XAD 7HP	550	13
Dowex 1x4	80	47

* See Appendix G for these results in chromatogram form

Dynamic Binding Capacity of TCEP Using Microporous Resins

For this experiment, the dynamic binding capacity of TCEP with Dowex 1x4, Dowex 1x8, Dowex Marathon A2 and Diaion SK1B was tested. 10 ml of 2 mM TCEP was added to each resin. Similarly to the previous experiment, these results were compared to the control to determine whether TCEP bound.

At pH 7, Dowex 1x8 appeared to bind the tightest to TCEP followed by Dowex 1x4 and then Dowex Marathon A2. (Table 2) The same pattern followed for pH 6.5. At pH 6 Dowex Marathon A2 appeared to have the strongest binding to TCEP while Dowex 1x4 and Dowex 1x8 were tied. However, once again, there was a margin of error for these experiments and a definitive ranking could not be made. Ultimately, the take away from this is that all three resins tested bound tightly to TCEP at pH 7, 6.5 and 6.

At pH 7, the maximum absorbance increase of Diaion SK1B was 450 mAU. While this was lower than the control result of 500 mAU, we assumed it was within the margin of error and concluded Diaion SK1B did not bind to TCEP.

Table 2: Maximum Absorbance Increase of Dowex and Diaion Resins (TCEP)

Resin	pH	Maximum Absorbance Increase (mAU)
Dowex 1x4	7	100
	6.5	70
	6	60
Dowex 1x8	7	75
	6.5	25
	6	60
Dowex Marathon A2	7	125
	6.5	110
	6	40
Diaion SK1B	7	450

*See Appendix H for these results in chromatogram form

Dynamic Binding Capacity of mAb1

To ensure separation of TCEP and mAb1, TCEP must bind to the resin in the column, while mAb1 flows through. For this experiment, we determined the dynamic binding capacity of mAb1 using the microporous resins. Once again, a control was used to determine the maximum absorbance increase of the system (UV detector) after adding 10 mg of mAb1to the system if no binding occurred. We found this value to be 900 mAU.

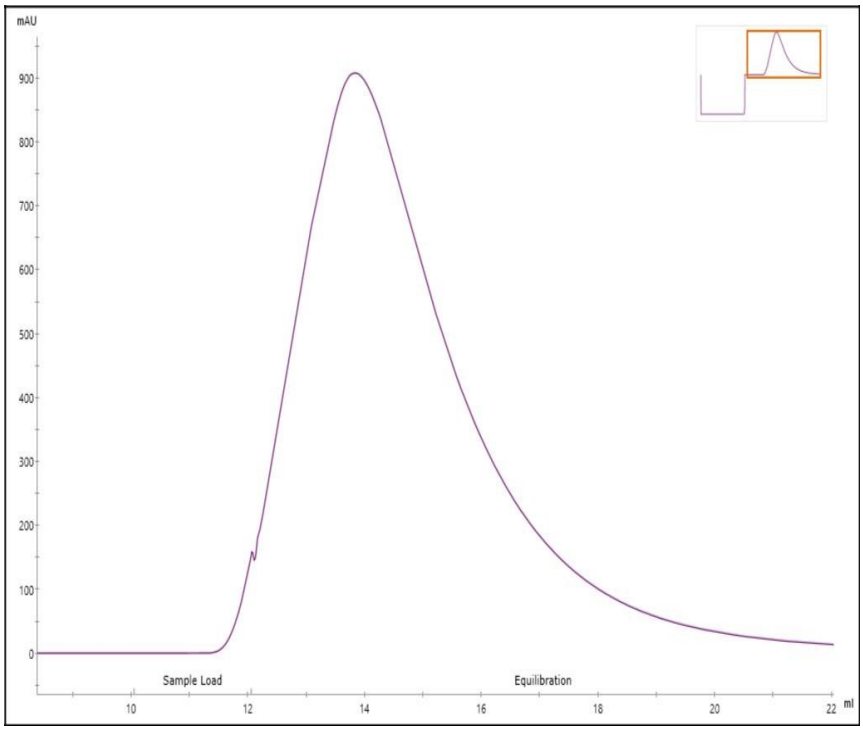

Figure 9: Chromatogram of control, mAb1, maximum absorbance increase of 900 mAU

For this experiment, 10 mg of mAb1 was added to four separate columns with Dowex 1x4, Dowex 1x8, Dowex Marathon A2 and Diaion SK1B. The protein did not bind to the Dowex resins at pH 7, 6.5 or 6. (Table 3) Similarly, it also did not bind to Diaion SK1B at pH 7. While the maximum absorbance increase did not always reach 900 mAU, the results were within the margin of error to conclude there was no binding.

Table 3: Maximum Absorbance Increase of Dowex and Diaion Resins (mAb1)

Resin	pH	Maximum Absorbance Increase (mAU)
Dowex 1x4	7	850
	6.5	900
	6	825
Dowex 1x8	7	825
	6.5	825
	6	825
Dowex Marathon A2	7	850
	6.5	875
	6	800
Diaion SK1B	7	825

*See Appendix I for these results in chromatogram form

Dynamic Binding Capacity of mAb1-TCEP Solution

Results from the previous experiments indicated that TCEP bound to Dowex 1x4, Dowex 1x8 and Dowex Marathon A2 but mAb1 did not. In order to make sure this remained true when TCEP and mAb1 were combined and mimic true conditions, we mixed them into a solution and the dynamic binding capacity was tested using Dowex 1x4 at pH 6.5.

The wavelength of 280 nm was used to test the dynamic binding capacity of mAb1, while 195 nm was used to test the dynamic binding capacity of TCEP. At 280 nm the maximum absorbance increase of the mAb1-TCEP solution was 800 mAU. After comparing it to the control from the previous mAb1 experiment, we concluded that mAb1 did not bind to Dowex 1x4 resin. At 195 nm the maximum absorbance increase of the mAb1-TCEP solution was 2150 mAU. This was much different from the control of the TCEP experiment where the maximum absorbance increase was 500 mAU. This data indicated that mAb1

was also being detected at 195 nm so we could not conclude whether TCEP was binding to Dowex 1x4.

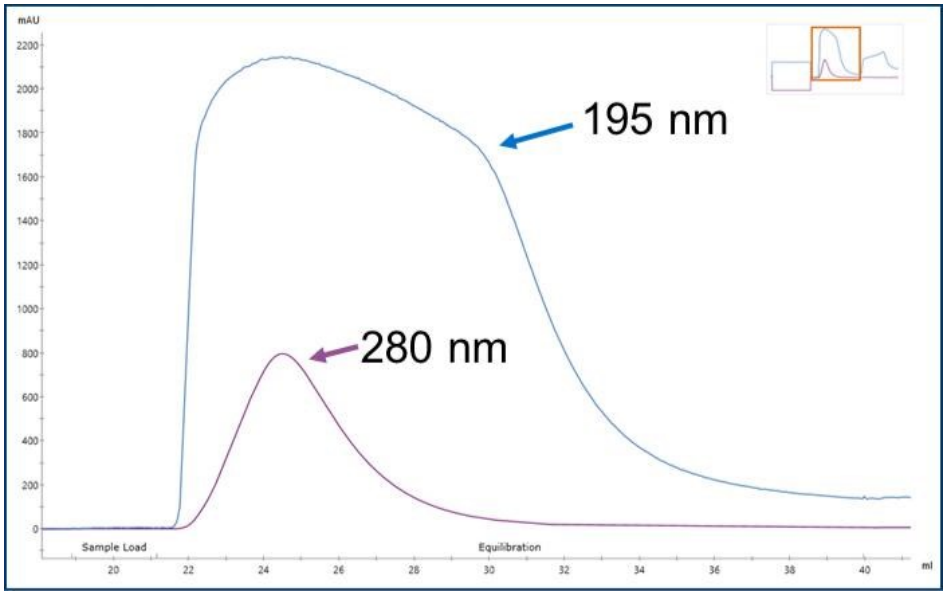

Figure 10: Chromatogram of mAb1-TCEP, Dowex 1x4, maximum absorbance increase of 2150 (195 nm) and 800 (280 nm)

ADC Synthesis

In order to test whether column chromatography using microporous resins was a feasible alternative to dialysis we had to go through the ADC synthesis process described in the methods section. The steps included sample preparation, reduction, dialysis or column chromatography, oxidation, conjugation, quenching of conjugation and analysis.

We first performed a control experiment and negative control. The control used the current ADC synthesis method, and resulted in successful conjugation. The negative control used the current ADC synthesis method without the dialysis step, and resulted in failed conjugation. These conclusions were determined using chromatograms from RPHPLC.

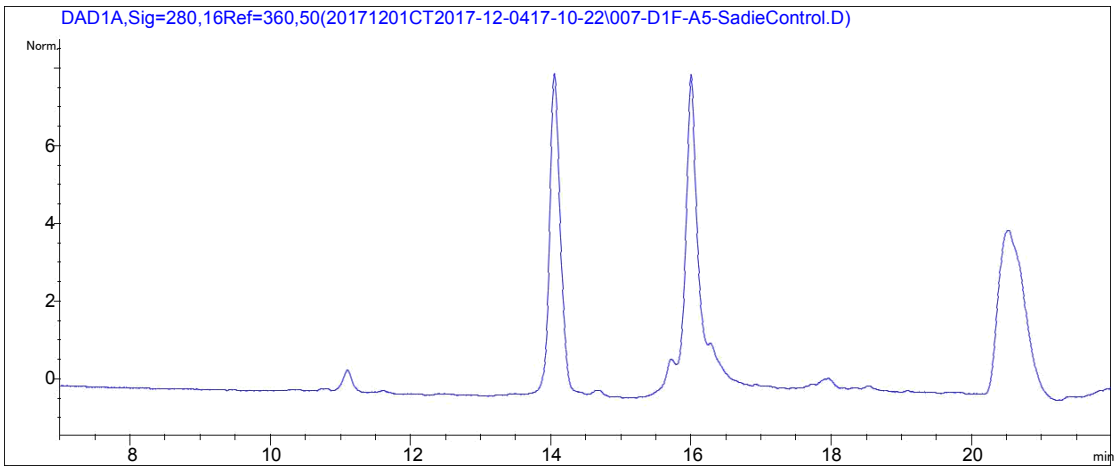

Figure 11: RPHPLC chromatogram of control - successful ADC synthesis, dialysis

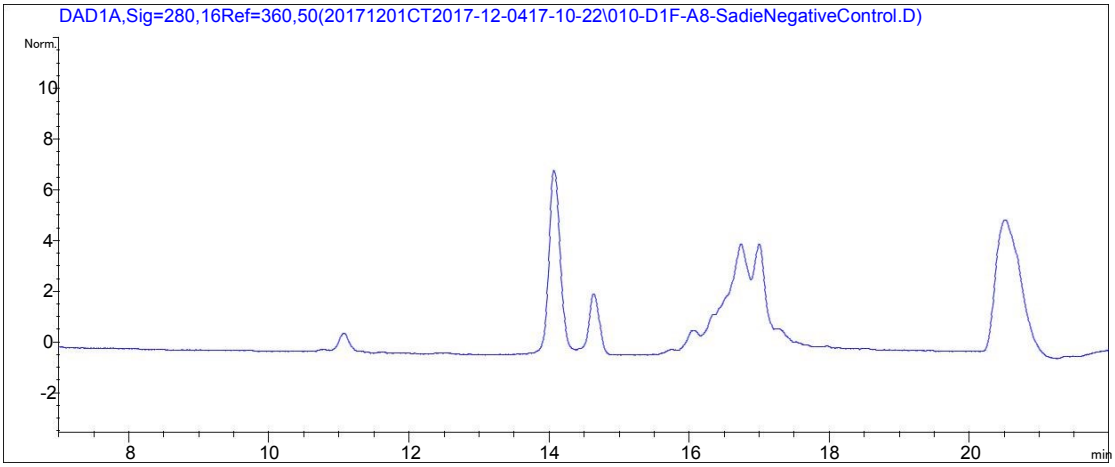

Figure 12: RPHPLC chromatogram of negative control - unsuccessful ADC synthesis, no dialysis or chromatography

ADC Synthesis with Column Chromatography

After the control, we performed another experiment following the current ADC synthesis method but implementing column chromatography instead of dialysis. These experiments were performed using Dowex 1x4, Dowex 1x8, Dowex Marathon A2 and Diaion SK1B. We compared these chromatograms to the control and negative control to determine whether conjugation was successful or not. For example, the results with Dowex 1x4 looked similar to the control, so we concluded synthesis was successful. (Figure 13)

The same was true for experiments with Dowex 1x8, Dowex Marathon A2 and Diaion SK1B. See Appendix J for chromatograms.

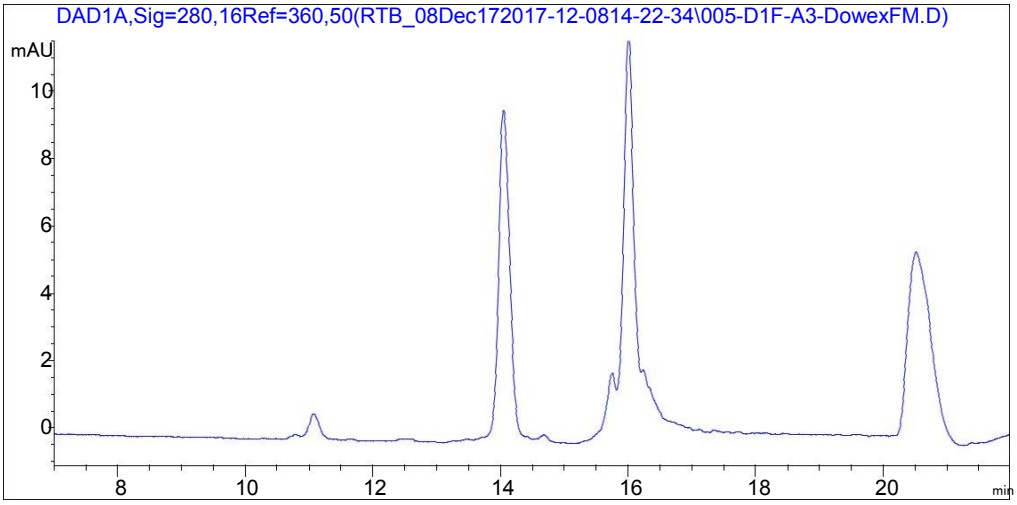

Figure 13: RPHPLC chromatogram - successful ADC synthesis, chromatography, Dowex 1x4

ADC Synthesis with Column Chromatography and no Sample Concentration

We first performed a negative control*. This experiment followed the same procedure as the control experiment described in the previous section but we removed the dialysis and sample concentration steps. We compared this chromatogram to the one from the previous control experiment and concluded the negative control* resulted in a failed conjugation.

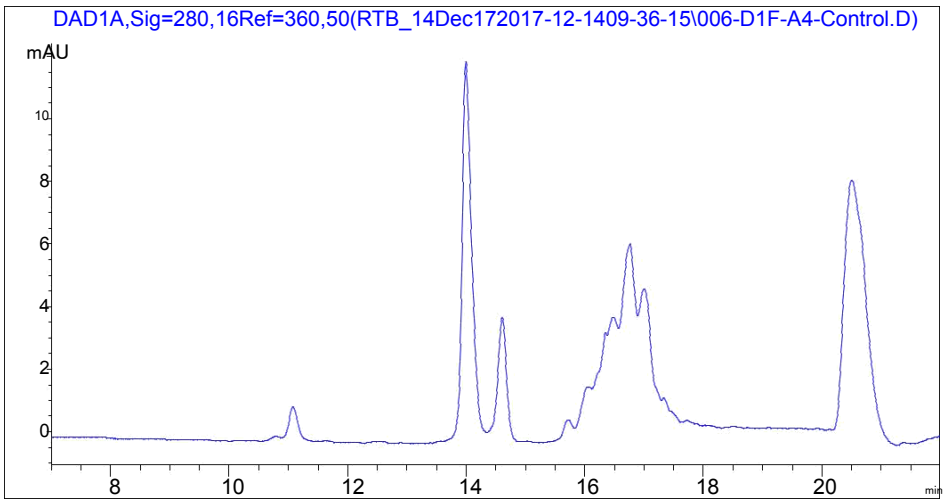

Figure 14: RPHPLC chromatogram of negative control - unsuccessful ADC synthesis, no dialysis or chromatography*

After the control, we performed another experiment following the current ADC synthesis method but implementing column chromatography instead of dialysis and removing the sample concentration step. These experiments were performed using Dowex 1x8, Dowex Marathon A2 and Diaion SK1B. We compared these chromatograms to the control and negative control* to determine whether conjugation was successful or not. The results with Dowex Marathon A2 looked similar to the control, so we concluded synthesis was successful. (Figure 15) The same was true for the experiment with Diaion SK1B. The result using Dowex 1x8 looked similar to the negative control* so we concluded ADC synthesis was not successful. See Appendix K for chromatograms.

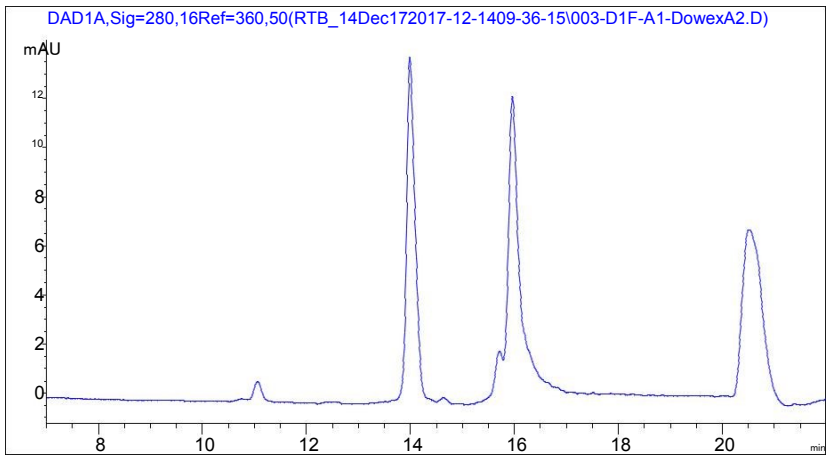

Figure 15: RPHPLC chromatogram - successful ADC synthesis, chromatography, Dowex Marathon A2

Discussion

The goal of this project was to implement column chromatography in flow through for the removal of TCEP using microporous resins. In order to do this, we first tested the dynamic binding capacity of TCEP. After selecting top performing resins, we tested the dynamic binding capacity of mAb1. Next, we tested the dynamic binding capacity of a mAb1-TCEP mixture. Finally, we performed antibody drug conjugate synthesis while implementing column chromatography instead of dialysis.

Dynamic Binding Capacity of TCEP

We started by testing a variety of resins for their ability to bind to TCEP. The first resin we tested was Amberlite FPX66. This is a macroporous adsorbent with an aromatic polymer backbone. We found that this resin did not bind to TCEP, most likely because it's comprised of a hydrophobic backbone. Amberlite XAD 7HP, another macroporous resin also did not bind to TCEP. This resin is moderately polar, with an acrylic resin backbone. Its main uses are to remove non polar compounds from aqueous solutions and slightly polar compounds from non-polar solvents. However, our TCEP solution did not meet these conditions, thus the resin was unable to adsorb and remove TCEP.

Amberlite FPA 98Cl resin, is a macroporous strong base anion exchanger and did show some binding capacity to TCEP. This makes sense because the TCEP solution was negatively charged. Dowex 1x4 resin showed the largest amount of TCEP binding. Like Amberlite FPA 98Cl, Dowex 1x4 is also a strong base anion exchanger. This resin is classified as a microporous resin, while Amberlite FPA 98Cl is a macroporous resin. Dowex 1x4 probably bound more strongly to TCEP because its pores are smaller.

Dynamic Binding of TCEP Using Microporous Resins

After identifying Dowex 1x4 as the resin with the highest TCEP binding capacity, we wanted to test the dynamic binding capacity of other similar resins. We chose Dowex 1x8 and Dowex Marathon A2, which are both micrporous strong anion exchangers. As a negative control, we also tested Diaion SK1B, which is a microporous strong cation exchanger.

At pH 7, 6.5 and 6 TCEP is expected to have a negative charge. This agrees with our results because TCEP was able to bind to Dowex 1x4, Dowex 1x8 and Dowex Marathon A2, which have positively charged functional groups. Conversely, it was not able to bind to Diaion SK1B resin, which has a negatively charged functional group.

Generally, as the pH of the TCEP solution was lowered, the binding capacity increased. Overall, for the anion exchange resins, it was clear that there was substantial binding at all pH levels.

Dynamic Binding Capacity of mAb1

From the previous experiments, we determined that TCEP could bind to charged microporous resins. To ensure separation, it was essential that our protein did not bind to these resins. Consequently, we determined the dynamic binding capacity of mAb1 using Dowex 1x4, Dowex 1x8, Dowex Marathon A2 and Diaion SK1B.

At pH 7, 6.5 and 6 mAb1 is expected to have a positive charge. This agrees with our results because mAb1 did not bind to Dowex 1x4, Dowex 1x8 and Dowex Marathon A2, which have positively charged functional groups. It also was not able to bind to Diaion SK1B resin, even though it has a negatively charged functional group. This is because Diaion SK1B is a microporous resins and its pores are too small for protein to enter.

Dynamic Binding Capacity of mAb1-TCEP Solution

Based on the experiments completed so far, TCEP appeared to bind to the negatively charged microporous resins while mAb1 did not. We combined TCEP and mAb1 into a solution to mimic true conditions. This also allowed us to test whether the previous results still held true after the two components were mixed.

We performed this experiment at pH 6.5 and used Dowex 1x4 resin. At this pH level TCEP is expected to be negatively charged while mAb1 is expected to be positively charged. At 280 nm, the wavelength used to measure proteins, we saw that mAb1 did not bind to the resin while in solution. At 195 nm, the wavelength used to measure TCEP, we could not determine whether TCEP was binding. We found that protein was also absorbed at 195 nm, so the reading we got was of the mAb1-TCEP solution. However, we hypothesized that TCEP was still binding.

ADC Synthesis using Column Chromatography

In order to determine whether column chromatography could separate TCEP and mAb1 we performed ADC synthesis. For our control, we followed the current conjugation method to demonstrate successful synthesis. For our negative control, we also used the current conjugation method but removed the dialysis step. This showed us what an unsuccessful synthesis looks like. Using RPHPLC, we compared the difference in successful vs unsuccessful synthesis chromatograms.

We then tested our new method; this followed the current synthesis method but replaced the dialysis step with column chromatography. We achieved successful synthesis using Dowex 1x4, Dowex 1x8, Dowex Marathon A2 and Diaion SK1B. Based on the results of our previous experiments, we expected this method to work with the Dowex resins. However, in past experiments, Diaion SK1B did not bind to TCEP. We hypothesized that the sample concentration step may have removed TCEP from the solution.

ADC Synthesis with Column Chromatography and no Sample Concentration

We performed another ADC synthesis experiment to test whether the sample concentration step caused the successful results while using Diaion SK1B. First we performed another negative control*, following the current synthesis method while removing the dialysis and sample concentration steps. This showed us what an unsuccessful conjugation looks like.

We then tested our new method; this followed the current synthesis method but replaced the dialysis step with column chromatography and removed the sample concentration step. We achieved successful synthesis using Dowex Marathon A2 and Diaion SK1B. Synthesis was unsuccessful using Dowex 1x8 resin. These results did not align with our hypothesis. We expected positive results with Dowex Marathon A2 and Dowex 1x8, but negative results with Diaion SK1B. The most probable explanation for these results is human error. There's a possibility that the Dowex 1x8 and Diaion SK1B resins were switched or mislabeled somewhere in the process. However, due to time constraints we were unable to repeat this experiment.

Further experiments are needed to determine the cause of this discrepancy. These experiments should be repeated in triplicate. Furthermore, experiments using this procedure should be performed using Dowex 1x4. Regardless of this variability, we have shown that microporous resins do remove TCEP from mAb1-TCEP solutions.

Conclusion

Overall, this project proved that TCEP removal using column chromatography in flow through mode is possible. Our first experiment helped us identify resins with a strong binding capacity to TCEP. Next, we performed experiments to ensure these resins did not also bind to mAb1. Finally, we performed antibody drug conjugate synthesis to test our new method. By achieving successful synthesis while implementing column

chromatography, we demonstrated this method is a promising alternative to dialysis. This will be particularly interesting for high-throughput conjugation screening applications, as it allows a rapid intermediate buffer exchange after the reduction step. As discussed in the previous section, further experiments are still warranted to delineate robustness of the method.

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Appendix A: Methods - Dynamic Binding Capacity of TCEP

Amberlite FPA 98Cl

A 1.2 ml column was packed with FPA 98 Cl resin, a macroporous strong anion exchanger. The method conditions and buffers are as follows.

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	2
Sample Load	2 mM TCEP	20	2
Regeneration	10% NaCl, 4% NaOH	10	.3
Column wash	Water for injection (WFI)	10	.5

*1 Column volume = 1.2 ml

Amberlite XAD 7HP

A 1.4 ml column was packed with Amberlite XAD 7HP resin, a macroporous non functionalized adsorbent. The method conditions and buffers are as follows.

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	2
Sample Load	2 mM TCEP	30	2
Regeneration	.1 N NaOH	10	.5
Column wash	Water for injection (WFI)	10	.5

*1 Column volume = 1.4 ml

Dowex 1x4

A 1.9 ml column was packed with Dowex 1x4 resin, a microporous strong anion exchanger. The method conditions and buffers are as follows.

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	2
Sample Load	2 mM TCEP	25	2
Elution	1M NaCl	15	2
Regeneration	10% NaCl, 4% NaOH	15	.5
Column wash	Water for injection (WFI)	10	.4

*1 Column volume = 1.9 ml

Appendix B: Methods - Dynamic Binding Capacity of TCEP Using Microporous Resins

Dowex 1x8

A 1.4 ml column was packed with Dowex 1x8 resin, a microporous strong anion exchanger. The method conditions and buffers are as follows.

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	2
Sample Load	2 mM TCEP	7.09	2
Equilibration	20 mM Phosphate	10	2
Regeneration	.1 N NaOH	5	1.31

*1 Column volume = 1.4 ml

Dowex Marathon A2

A 1.5 ml column was packed with Dowex Marathon A2 resin, a microporous strong anion exchanger. The method conditions and buffers are as follows.

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	2
Sample Load	2 mM TCEP	6.71	2
Equilibration	20 mM Phosphate	10	2
Regeneration	1 N NaOH	5	1.31
Column wash	20 mM Phosphate	5	.4

*1 Column volume = 1.5 ml

Diaion SK1B

A 1.4 ml column was packed with Diaion SK1B resin, a microporous strong cation exchanger. The method conditions and buffers are as follows.

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	2
Sample Load	2 mM TCEP	7.09	2
Equilibration	20 mM Phosphate	10	2
Regeneration	2 M HCl	5	1.31

*1 Column volume = 1.4 ml

Appendix C: Methods - Dynamic Binding Capacity of mAb1

Dowex 1x8

A 1.4 ml column was packed with Dowex 1x8 resin. The method conditions and buffers are as follows.

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	4.62 mg/ml mAb1	1.53	.3
Equilibration	20 mM Phosphate	10	1.31
Regeneration	.1 N NaOH	5	1.31

*1 Column volume = 1.4 ml

Dowex Marathon A2

A 1.5 ml column was packed with Dowex Marathon A2 resin. The method conditions and buffers are as follows.

For pH 7 and 6

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	4.53 mg/ml mAb1	1.5	.3
Equilibration	20 mM Phosphate	10	1.31
Regeneration	1 N NaOH	5	1.31
Column Wash	20 mM Phosphate	5	1.31

*1 Column volume = 1.5 ml

For pH 6.5

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	5.66 mg/ml mAb1	1.3	.3
Equilibration	20 mM Phosphate	10	1.31
Regeneration	1 N NaOH	5	1.31
Column Wash	20 mM Phosphate	5	1.31

*1 Column volume = 1.5 ml

Diaion SK1B

A 1.4 ml column was packed with Diaion SK1B resin. The method conditions and buffers are as follows.

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	4.62 mg/ml mAb1	1.53	.3
Equilibration	20 mM Phosphate	10	1.31
Regeneration	2 M HCl	5	1.31

*1 Column volume = 1.4 ml

Appendix D: Methods – ADC Synthesis RPHPLC Conditions

Time (min)	%A (Water + Trifluoroacetic Acid)	%B (Acetonitrile + Trifluoroacetic Acid)
0	80	20
3	80	20
20	60	40
22	5	95
26	5	95
30	80	20

Appendix E: Methods – ADC Synthesis with Column Chromatography

Dowex 1x4

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	5.49 mg/ml mAb1	1.35	.26
Equilibration	20 mM Phosphate	10	1.31
Regeneration	.1 N NaOH	5	1.31

*1 Column volume = 1.8 ml

Dowex 1x8

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	9.38 mg/ml mAb1	1	.26
Equilibration	20 mM Phosphate	10	1.31
Regeneration	.1 N NaOH	5	1.31

*1 Column volume = 1.4 ml

Dowex Marathon A2

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	5.39 mg/ml mAb1	1.8	.26
Equilibration	20 mM Phosphate	10	1.31
Regeneration	1 N NaOH	5	1.31

*1 Column volume = 1.7 ml

Diaion SK1B

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	5.39 mg/ml mAb1	1.75	.26
Equilibration	20 mM Phosphate	10	1.31
Regeneration	2 M HCl	5	1.31

*1 Column volume = 1.4 ml

Appendix F: Methods - ADC Synthesis with Column Chromatography and no Sample Concentration

Dowex 1x8

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	10.25 mg/ml mAb1	1	.26
Equilibration	20 mM Phosphate	10	1.31
Regeneration	.1 N NaOH	5	1.31

*1 Column volume = 1.3 ml

Dowex Marathon A2

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	10.25 mg/ml mAb1	1	.26
Equilibration	20 mM Phosphate	10	1.31
Regeneration	1 N NaOH	5	1.31

*1 Column volume = 1.3 ml

Diaion SK1B

	Sample/Buffer	Column Volume	Flow rate (ml/min)
Equilibration	20 mM Phosphate	10	1.31
Sample Load	10.25 mg/ml mAb1	1	.26
Equilibration	20 mM Phosphate	10	1.31
Regeneration	2 M HCl	5	1.31

*1 Column volume = 1.4 ml

Appendix G: Results - Dynamic Binding Capacity of TCEP

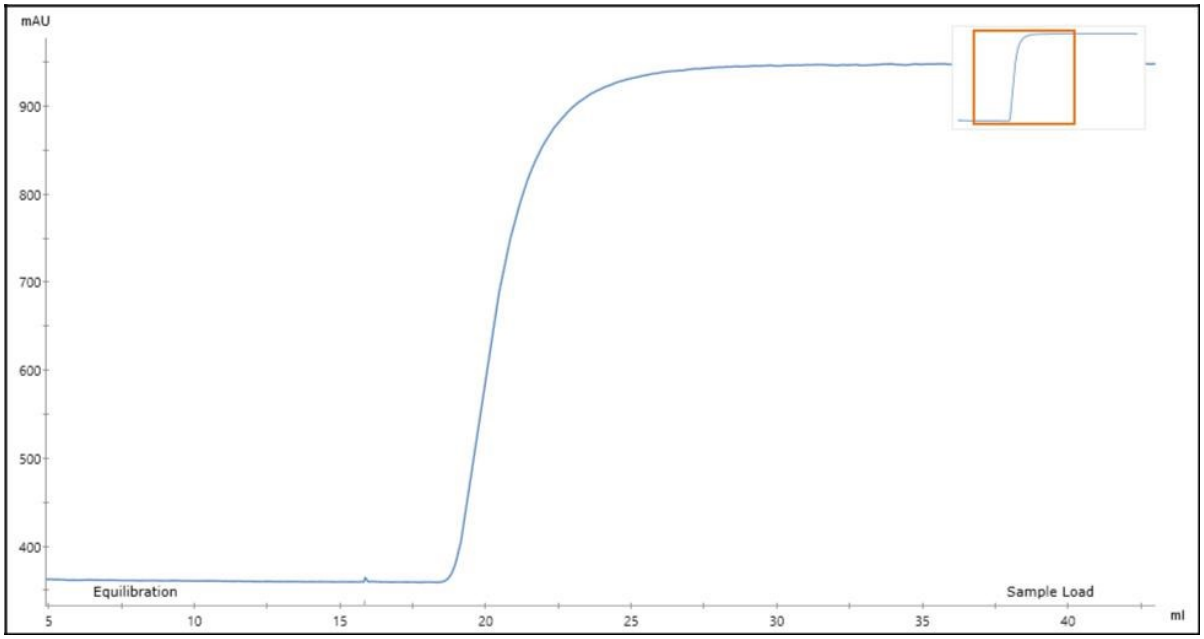

Figure 16: Chromatogram of TCEP, Amberlite FPX66, maximum absorbance increase of 575 mAU

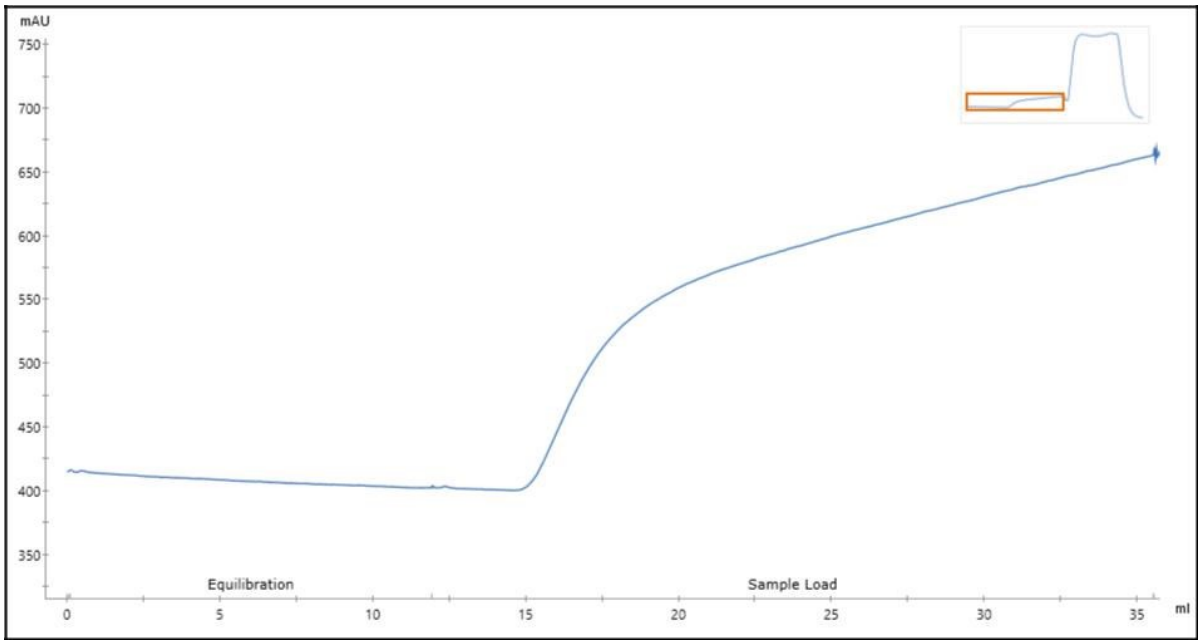

Figure 17: Chromatogram of TCEP, Amberlite 98Cl, maximum absorbance increase of 275 mAU

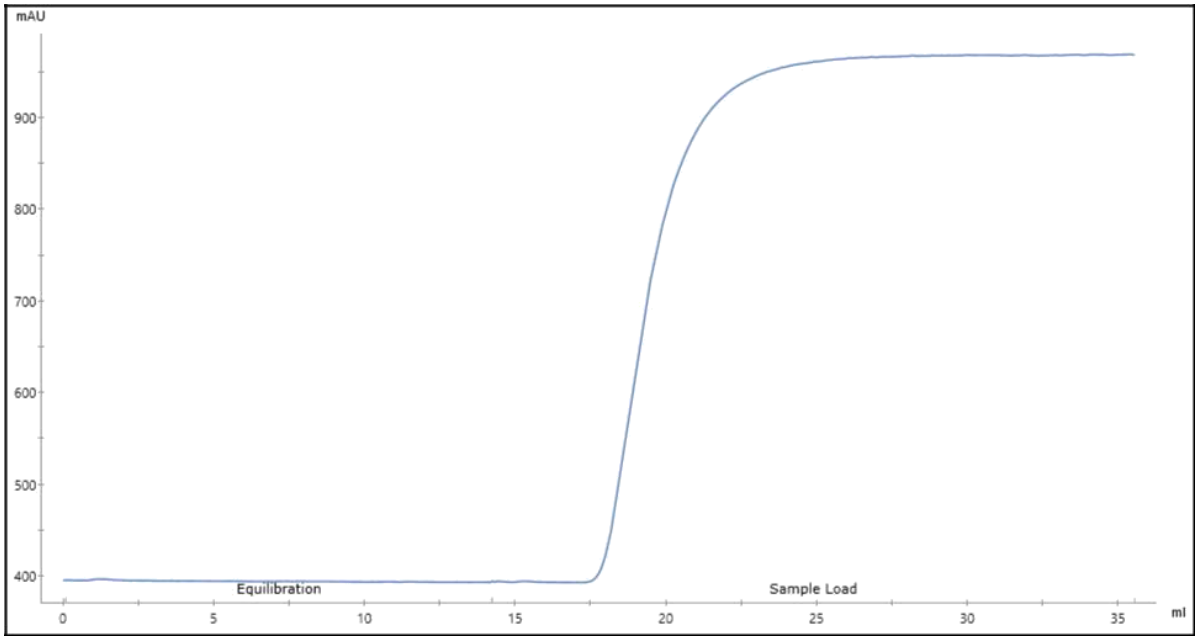

Figure 18: Chromatogram of TCEP, Amberlite XAD 7HP, maximum absorbance increase of 550 mAU

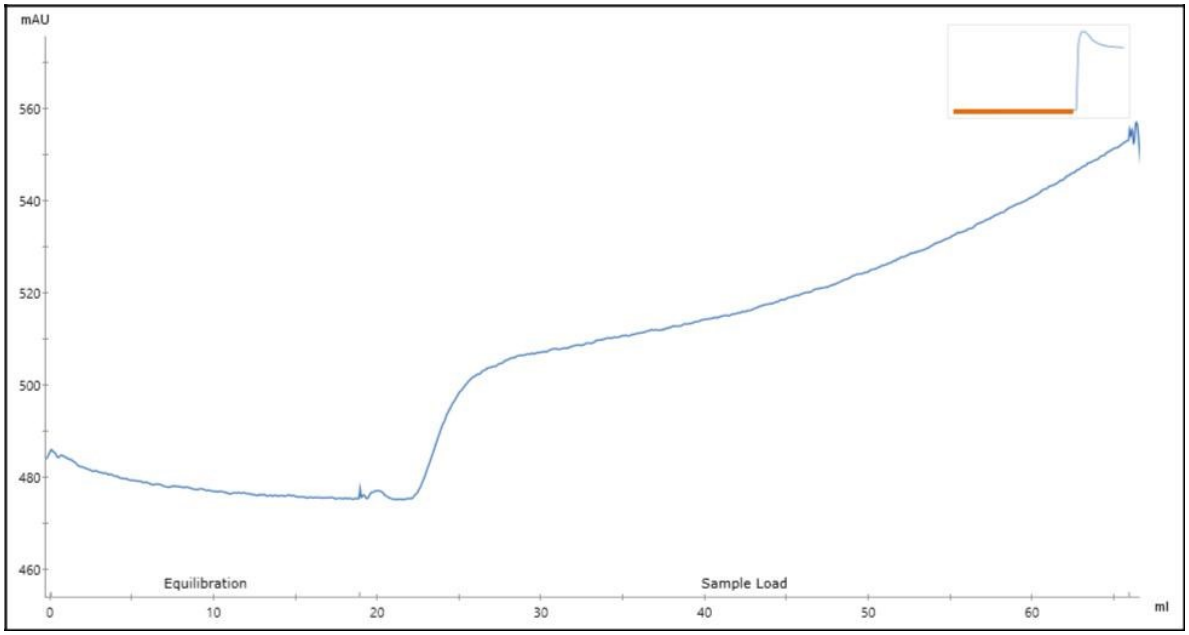

Figure 19: Chromatogram of TCEP, Dowex 1x4, maximum absorbance increase of 80 mAU

Appendix H: Results - Dynamic Binding Capacity of TCEP Using Microporous Resins

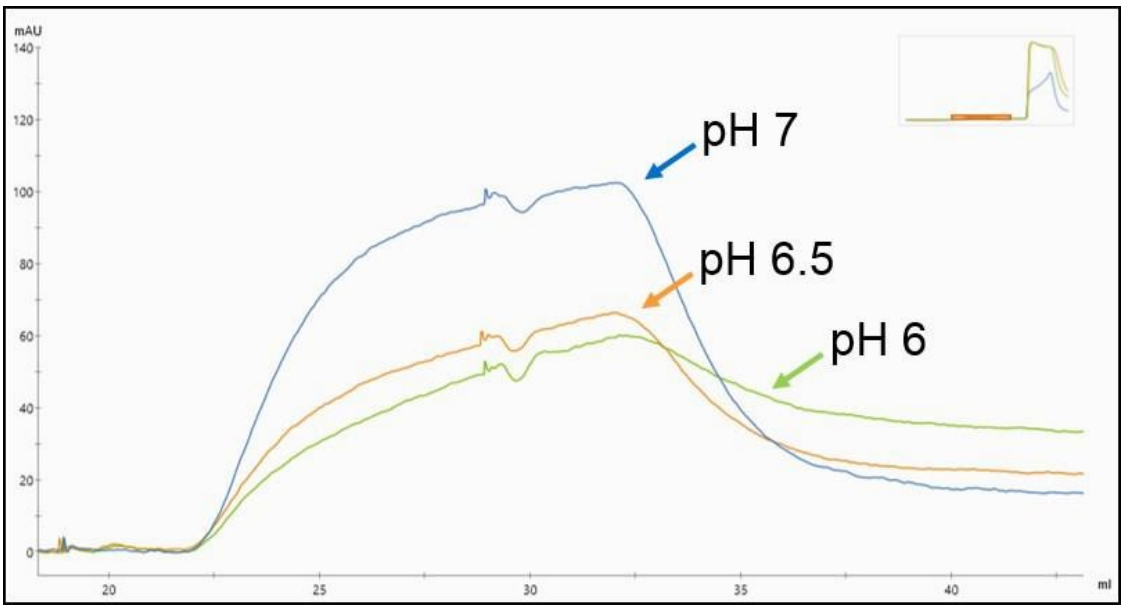

Figure 20: Chromatogram of TCEP, Dowex 1x4, pH 6, 6.5, 7, maximum absorbance increase of 60, 70 and 100 mAU respectively

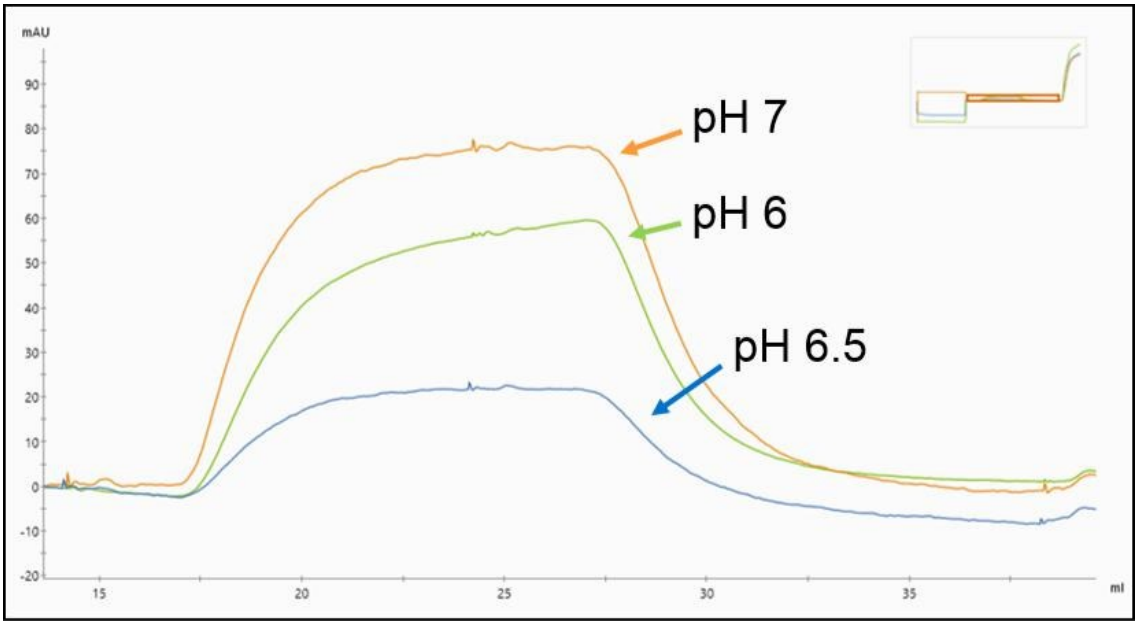

Figure 21: Chromatogram of TCEP, Dowex 1x8, pH 6, 6.5, 7, maximum absorbance increase of 60, 25 and 75 mAU respectively

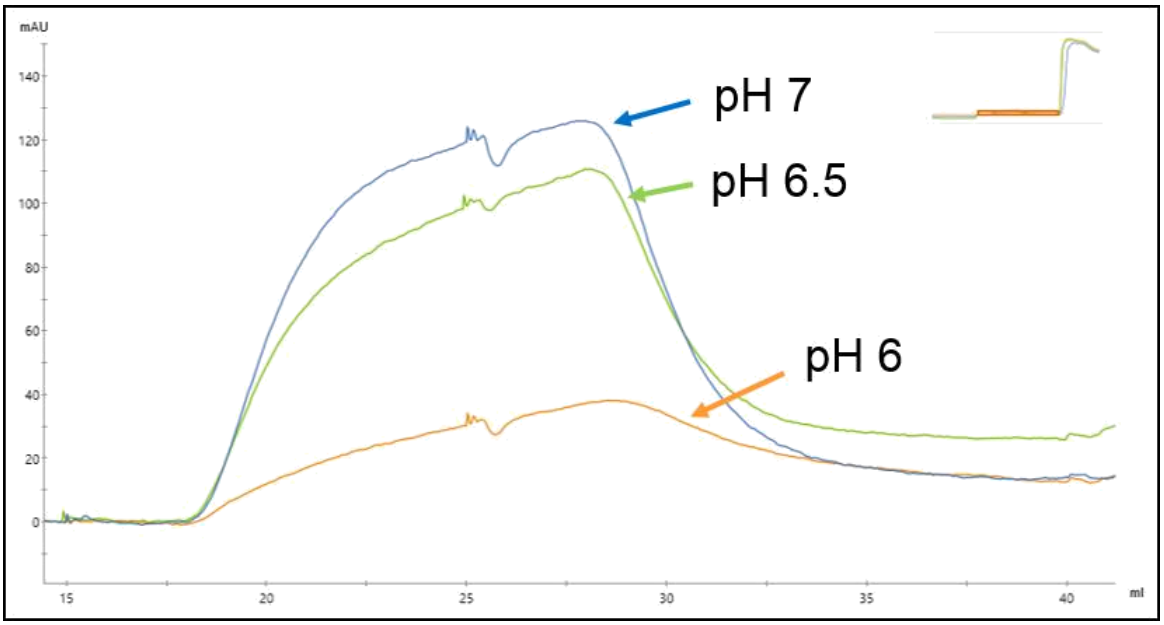

Figure 22: Chromatogram of TCEP, Dowex Marathon A2, pH 6, 6.5, 7, maximum absorbance increase of 40, 110 and 125 mAU respectively

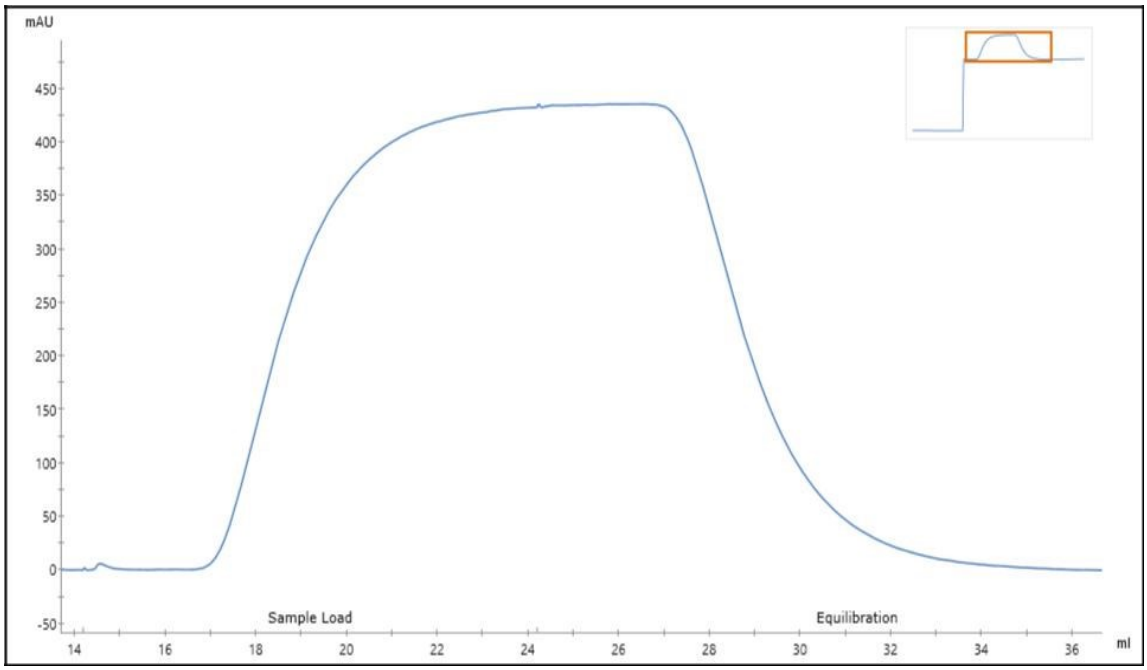

Figure 23: Chromatogram of TCEP, Diaion SK1B, pH 7, maximum absorbance increase of 450 mAU

Appendix I: Results - Dynamic Binding Capacity of mAb1

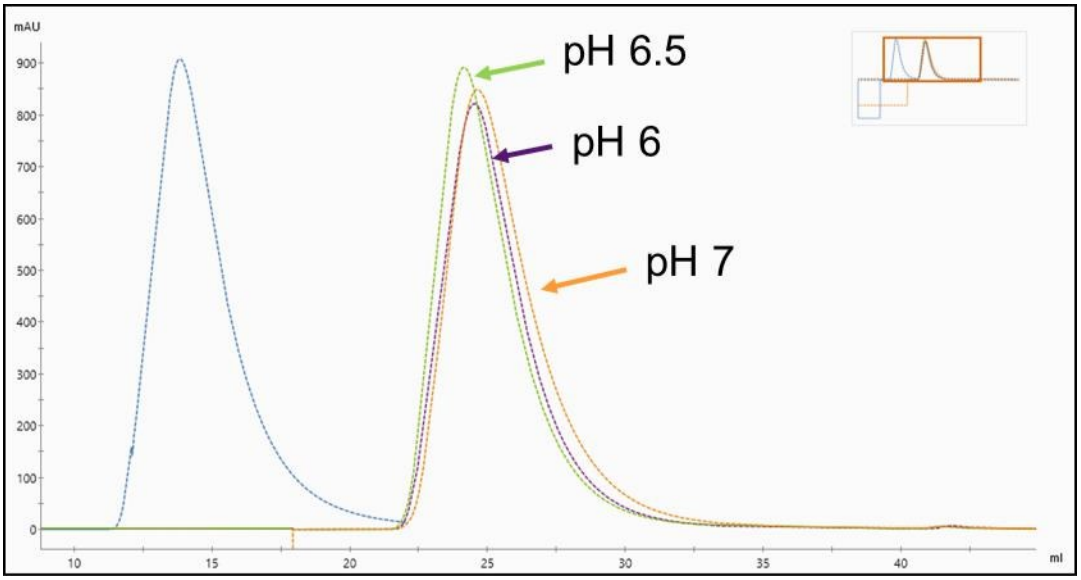

Figure 24: Chromatogram of mAb1, Dowex 1x4, pH 6, 6.5, 7, maximum absorbance increase of 825, 900 and 850 mAU respectively

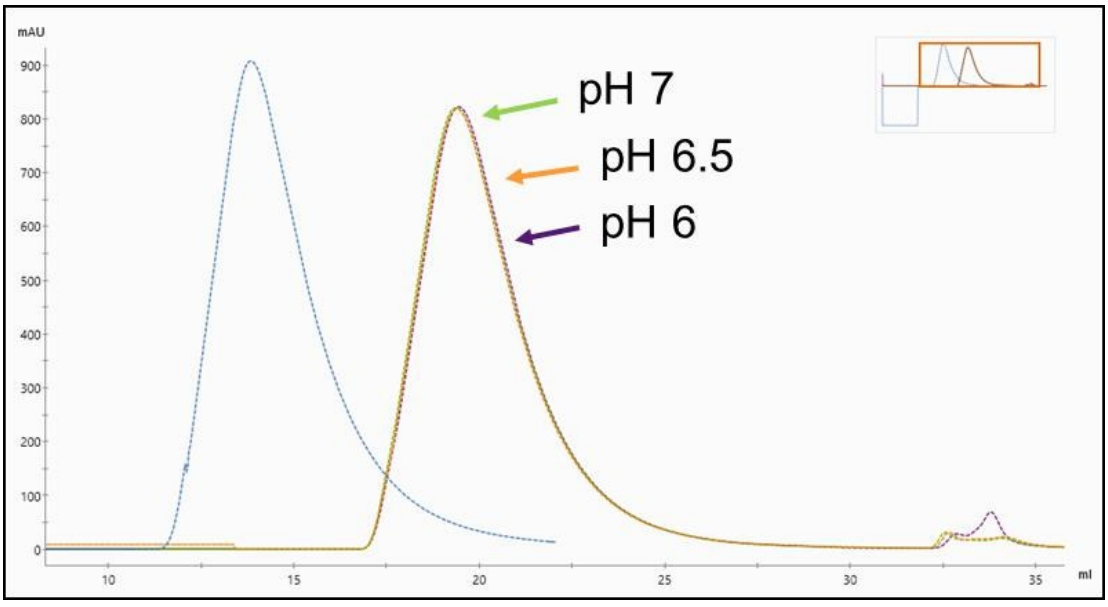

Figure 25: Chromatogram of mAb1, Dowex 1x8, pH 6, 6.5, 7 maximum absorbance increase of 825 mAU

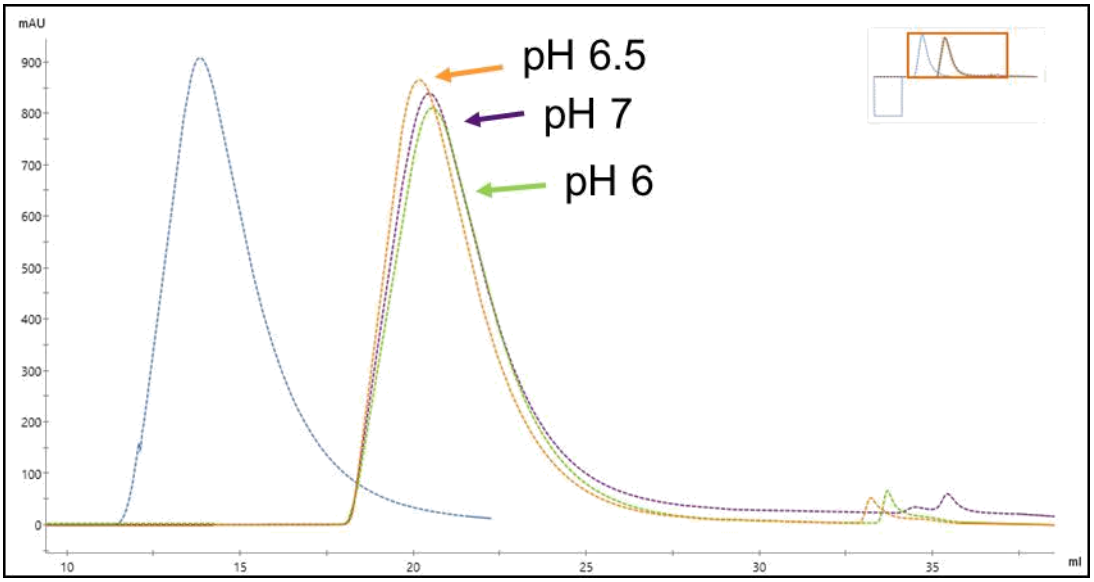

Figure 26: Chromatogram of mAb1, Dowex Marathon A2, pH 6, 6.5, 7, maximum absorbance increase of 800, 875 and 850 mAU respectively

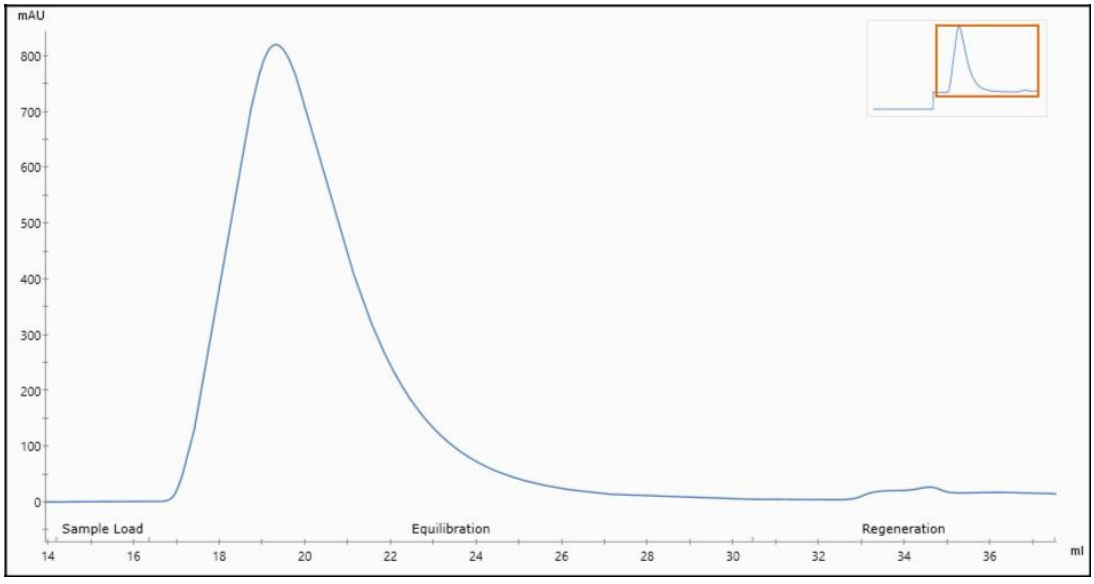

Figure 27: Chromatogram of mAb1, Diaion SK1B, pH 7, maximum absorbance increase of 825 mAU

Appendix J: Results – ADC Synthesis with Column Chromatography

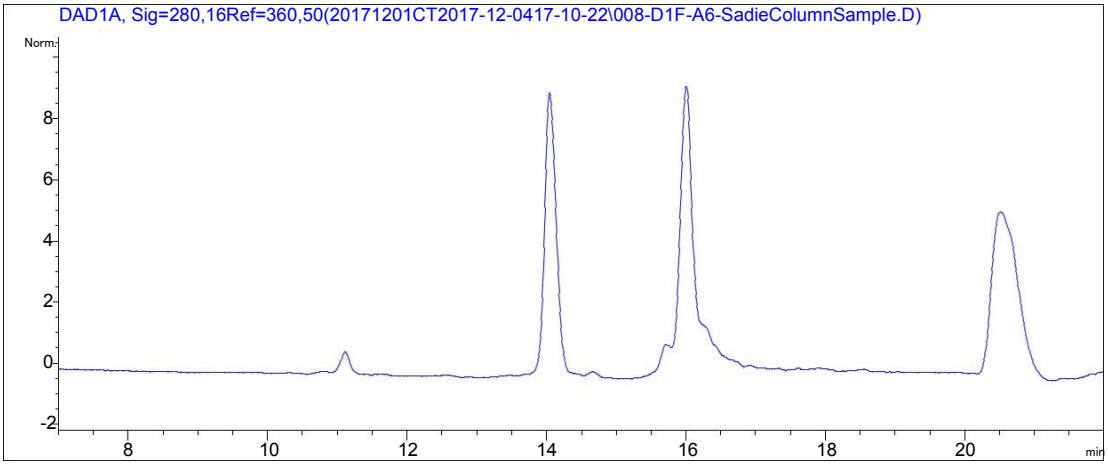

Figure 28: RPHPLC chromatogram - successful ADC synthesis, chromatography, Dowex 1x8

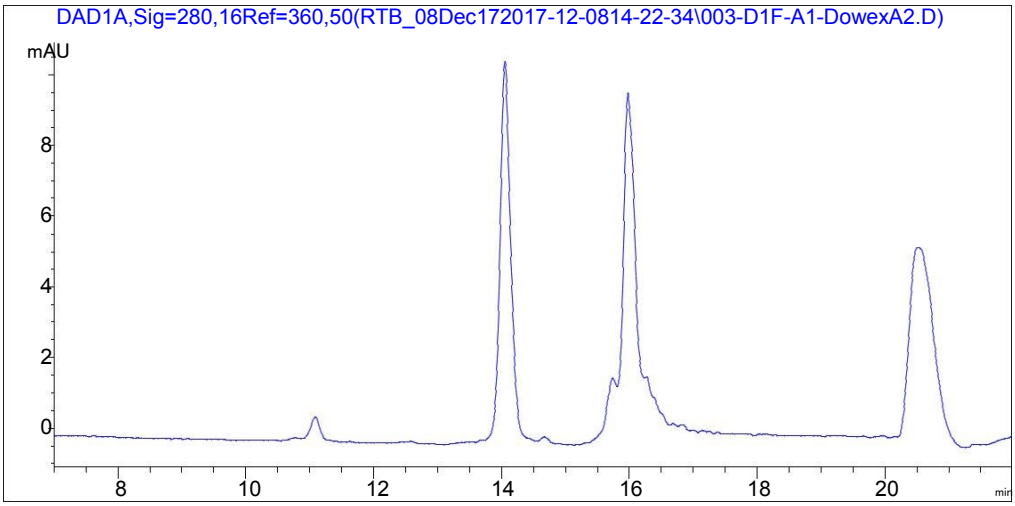

Figure 29: RPHPLC chromatogram - successful ADC synthesis, chromatography, Dowex Marathon A2

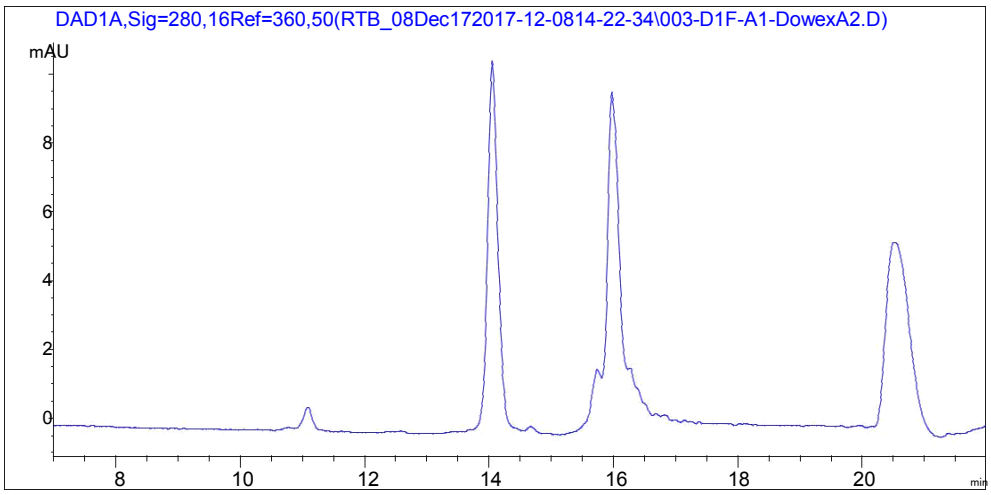

Figure 30: RPHPLC chromatogram - successful ADC synthesis, chromatography, Diaion SK1B

Appendix K: Results – ADC Synthesis with Column Chromatography and no Sample Concentration

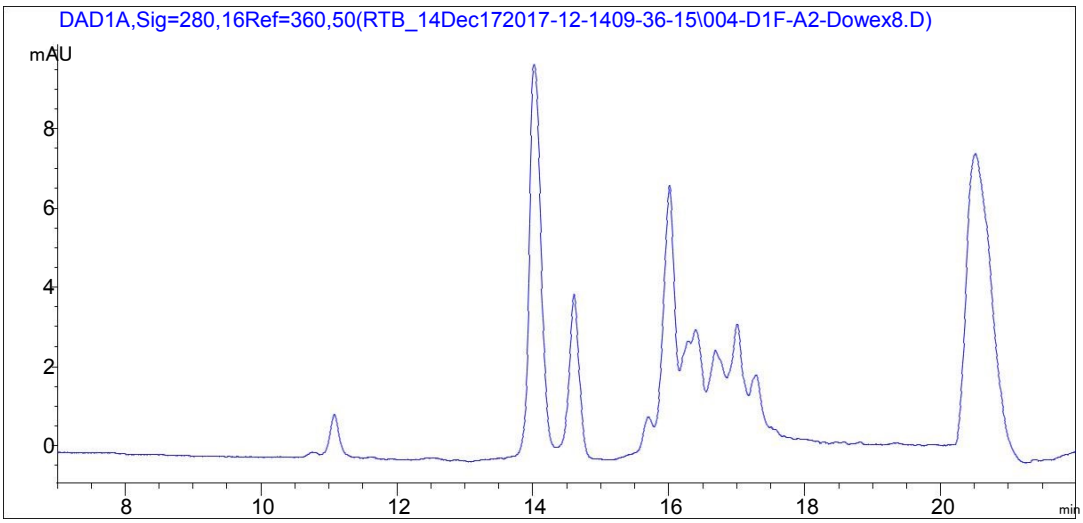

Figure 31: RPHPLC chromatogram - unsuccessful ADC synthesis, chromatography, Dowex 1x8

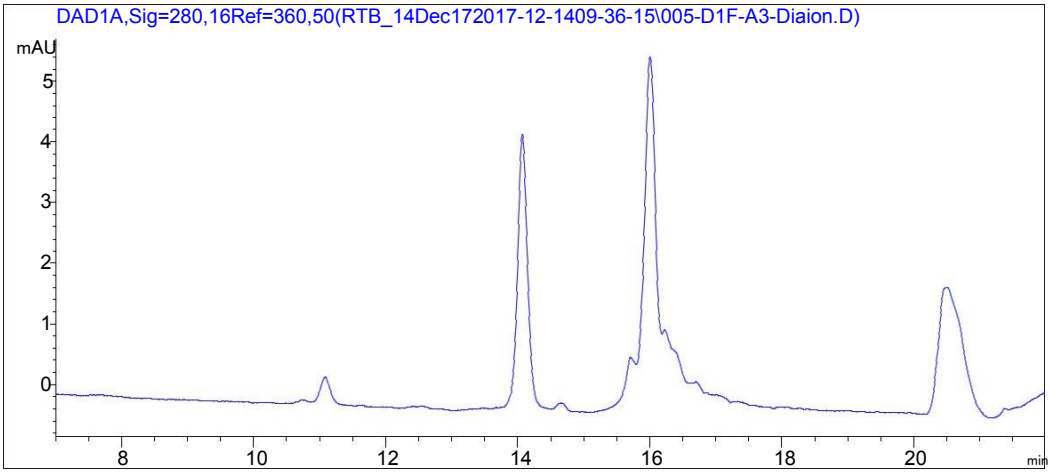

Figure 32: RPHPLC chromatogram - successful ADC synthesis, chromatography, Diaion SK1B

Resume

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Education

M.S. Chemical and Biomolecular Engineering | May 2018 | Johns Hopkins University

B.S. Chemical and Biomolecular Engineering | May 2017 | Johns Hopkins University

Skills

Laboratory: HPLC, UPLC, AKTA, Column packing, Depth filtration, Dialysis membranes
Software: UNICORN, OpenLAB, Empower, JMP, ELN, LIMS, MATLAB/Simulink, Microsoft Office Suite

Work Experience

Antibody Drug Conjugate Co-Op, Protein Purification | MedImmune | June 2017 – December 2017

Increasing the efficiency of antibody drug conjugate synthesis

Project 1: Improving the reduction & post-reduction purification method for the synthesis of site-specific antibody drug conjugates using DTBA and cation exchange displacement chromatography

- Performed high-throughput screening for chromatographic resin optimization
- Chose top performing resins and tested the binding capacity of DTBA, mAb and mAb-DTBA mixtures
- Developed an assay to quantify residual DTBA levels in mAb-DTBA mixtures

Project 2: Improving the post-reduction purification method for the synthesis of site-specific antibody drug conjugates using column chromatography on microporous resins

- Tested the binding capacity of TCEP, mAb, and mAb-TCEP solutions across a range of resins at multiple pH levels
- Tested the binding capacity of TCEP using a depth filter
- Achieved successful conjugation while implementing column chromatography method
- Demonstrated column chromatography removes as much TCEP as the current removal method while saving time

Automation Intern, Analytics | Regeneron Pharmaceuticals | June 2016 – August 2016

Designed and tested UPLC methods to automate amino acid assays
Optimized derivatization conditions using Design of Experiment to test factors

of interest
Conducted robustness studies to determine the most accurate and repeatable operating conditions
Determined automation achieved a time savings of 3 minutes/sample
Managed daily titer runs and reported data using LIMS

Protein Engineering Researcher | Ostermeier Laboratory, JHU| January 2015 – May 2016

Identified glycine betaine (GB)-activated TALE colonies from a combinatorial library expressed in *E. Coli* using positive and negative selection
Performed minimum inhibitory concentration assays on the colonies from selection to investigate the MIC shift for ampicillin in the presence and absence of GB
Determined the relationship between the addition of multiple TALE binding sites and gene expression
Evaluated the effect of GB on fluorescent protein expression